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Thanks.
Jennifer Hunt
Patent Examiner, Art Unit 1642
CM1-8D06
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Sit -Sp cific Conjugation of an Enzyme and an Antibody Fragm nt

Raymond C. Werlen,*,† Mervi Lankinen,† Keith Rose,† David Blakey,‡ Helen Shuttleworth,§ Roger Melton,§ and Robin E. Offord†

Département de Biochimie Médicale, Centre Médical Universitaire, 1 rue Michel Servet, CH 1211 Geneva 4, Switzerland, Cancer Research Department, Zeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK 10 4TG, U.K., and Division of Biotechnology, PHLS/CAMR, Porton Down, Salisbury, Wiltshire SP4 OJG, U.K. Received February 3, 1994®

A site-specific immunoconjugate was prepared between an $F(ab')_2$-like fragment of the monoclonal anti-CEA murine IgG1 A5B7 and a mutant of the dimeric enzyme carboxypeptidase G2 possessing an N-terminal Thr in place of Ala. First an aldehyde was introduced at the N-terminus of the enzyme by mild periodate oxidation and a residue of carbohydrazide was specifically introduced at the C-terminus of the truncated heavy chain of the $F(ab')_2$-like fragment by reverse proteolysis. Then the two modified proteins were conjugated by the formation of a hydrazone bond between the hydrazide and the aldehyde groups. The conjugate obtained retained both enzymic activity and antigen-binding capacity. The antigen-binding capacity was better than that of a similar conjugate made conventionally by random reaction with side chains.

INTRODUCTION

Antibody-directed enzyme prodrug therapy (ADEPT)[1] is a technique which uses a conjugate consisting of an antibody (or antibody fragment) bound to an enzyme to enhance the therapeutic benefit of chemotherapy by converting *in situ* a nontoxic prodrug to a toxic drug (for a recent review, see Senter et al. (1993)). At present, most immunoconjugates are prepared by introducing complementary reactive groups into the protein partners via acylation of lysine side chains with heterobifunctional reagents (for a recent review of this technology, see Brinkley (1992)). By controlling the extent of acylation and then purifying the conjugate according to its size, useful heterodimers can be prepared. However, the conjugates obtained through this approach consist generally of a mixture of isomers each linked through different residues, and each isomer may potentially have a different biological activity. Recently, techniques have been described which permit the introduction of a hydrazide group selectively at C-termini of proteins by reverse proteolysis (Rose et al., 1991; Fisch et al., 1992) and the introduction of an aldehyde group at the N-terminus of a protein (when this is occupied by Ser or Thr, see Scheme 1) through mild periodate oxidation of the 1,2 amino-ol characteristic of these residues when in the N-terminal position (Geoghegan and Stroh, 1992, and references cited therein). As shown in Scheme 2, hydrazide and aldehyde groups are able to react specifically with each other to give a hydrazone, and this can be exploited in the construction of protein conjugates (Rose et al., 1991). The combination of these two techniques allows the construction, via hydrazone formation, of homogeneous proteins with a new backbone (Gaertner et al., 1992) by head-to-tail conjugation without the need of any protecting groups. In the present paper, we have applied this approach to the preparation of a conjugate designed for ADEPT. We describe the conjugation of an $F(ab')_2$-like fragment of the monoclonal anti-CEA IgG1 murine antibody A5B7 to the enzyme carboxypeptidase G2 (CPG2), which is known to be able to convert nontoxic prodrugs into toxic drugs (Bagshawe et al., 1988). Conjugates of these two proteins have already been shown to be of interest for ADEPT (Sharma et al., 1991). Conjugation is achieved via the formation of a hydrazone bond between the C-terminus of the truncated heavy chain of the $F(ab')_2$ and the N-terminus of a mutant of the carboxypeptidase having an N-terminal threonine.

MATERIALS AND METHODS

Materials. Except where otherwise specified, solvent and reagents were of analytical grade or better, were obtained from commercial sources, and were used without further purification. The pH of solutions was adjusted at room temperature (about 22 °C). Urea was purified by passing an 8 M solution through a column packed with Serdolit MB3 (Serva) just prior to use. Lysyl endopeptidase (2.3 U/mg; amidase activity using N^{α}-benzoyl-DL-lysine-*p*-nitroanilide as substrate) from *Achromobacter lyticus* was obtained from Wako Pure Chemical Co. (Neuss, Germany). The A5B7 IgG1 murine antibody was obtained from CRC (Sutton, U.K.) as a 5.4 mg/mL solution in phosphate-buffered saline.

General Methods. The enzymic activity of CPG2 was assayed as previously described (Sherwood et al., 1985) except that the reaction was carried out at room temperature.

The binding activity of the conjugate was measured in a competitive binding assay using an alkaline phosphatase-labeled A5B7 standard. Briefly, 96-well ELISA plates were coated with CEA (0.1 μg/well) followed by the addition of mixtures of conjugate (10–0.5 μg/mL) and labeled A5B7 (1 μg/mL). After 90 min unbound antibody was removed and the amount of labeled A5B7 bound was determined by addition of *p*-nitrophenyl phosphate (Sigma) and measurement of the absorbance at 405 nm. The ability of the conjugate to compete with the labeled A5B7

† Centre Médical Universitaire.

‡ Zeneca Pharmaceuticals.

§ PHLS/CAMR.

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[1] Abbreviations: ADEPT, antibody-directed enzyme prodrug therapy; DAI, des-Ala[B30] porcine insulin; CPG2, carboxypeptidase G2; ESMS, electrospray ionization mass spectrometry; SDS–PA , sodium dodecyl sulfate polyacrylamide gel electrophoresis; AU, absorption unit; MES, morpholino ethyl sulfonate; CEA, carcino embryonic antigen; PCR, polymerase chain reaction.

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Scheme 1

$$H_2N\text{-}\underset{|}{\overset{\text{HO-CHR}}{\text{CH}}}\text{-CONH-}\mathit{PROTEIN}\text{-OH} + IO_4 \dashrightarrow O{=}CH\text{-C NH-}\mathit{PROTEIN}\text{-OH} + NH_3 + IO_3 + RCHO$$

$$(R = H,\ Ser;\ R = CH_3,\ Thr)$$

Scheme 2

$$\mathit{PROTEIN1}\text{-CONH-}NH_2 + O{=}CH\text{-}\mathit{PROTEIN2} \longrightarrow \mathit{PROTEIN1}\text{-CONH-N=CH-}\mathit{PROTEIN2} + H_2O$$

for binding to CEA was compared to intact unlabeled A5B7 to give a percent binding activity relative to unconjugated antibody.

Protein solutions were concentrated on Centriprep 10 or Centricon 10 devices following the instructions of the manufacturer (Amicon). Since glycerol could interfere with periodate oxidation, when necessary, the glycerol present as protecting agent for the membrane was removed by rinsing the concentrator three times with water and three times with the buffer to be used for the concentration and then by passing fresh buffer through the membrane twice prior to the concentration step.

Electrophoresis on polyacrylamide gels in SDS (SDS–PAGE) was performed on Pharmacia Phast system using 8–25% gradient gels, and proteins were detected with the silver nitrate procedure according to the recommendations of the manufacturer.

Desalting was performed on a Pharmacia FPLC system using a HR10/10 fast desalting column. The same system was used for gel filtration using a Superose 12 HR 10/30 column and for ion exchange chromatography with a Mono S HR5/5 column.

The samples for electrospray ionization mass spectrometry (ESMS) were dialyzed against 10 mM dithiothreitol. ESMS was performed as previously described (Vilaseca et al., 1993).

Preparation of the Threonine N-Terminal CPG2. The N-terminal Ala was replaced by a Thr using standard PCR technology (McPherson et al., 1993) with two mutagenic oligonucleotides: a 26bp oligonucleotide complementary to the DNA sequence immediately downstream of the CPG2 translational terminus, which introduced a mutagenic *Hind* III restriction site, and a second 30bp oligonucleotide complementary to the nucleotide sequence of the 5' end of CPG2 gene, which coded for the Ala to Thr codon change and also introduced a *Sca* I restriction site across the DNA coding for the peptidase cleavage site of the immature CPG2 (see Figure 1). The presence of these two unique restriction enzyme sites facilitated the cloning of the 1.15 kb (threonine mutant) CPG2 gene into the *Nae* I–*Hind* III sites of a specifically designed secretion vector: pMTL3223 (manuscript in preparation) to give the recombinant vector pCPS3. The mutant CPG2 was expressed in *E. coli* MC1061 transformed with pCPS3. Cultures were grown overnight in 1 L of L-broth containing 30 μg/mL of chloramphenicol. The cells were harvested by centrifugation (6000g for 20 min) and then resuspended in 10 mL of 10 mM sodium acetate pH 5.5 and sonicated (MSE soniprep 150, 3 × 20 s, amp 18 μm). The cell debris was removed by centrifugation (17000g for 45 min) and the supernatent containing CPG2 collected for further purification.

Purification f Mutant CPG$_2$. The enzyme was purified to homogeneity, as judged by SDS–PAGE (Figure 2, lane 8), from the c ll supernatant by a two-step ion-exchange chromatography method. The obtained mutant enzyme had a specific activity indistinguishable from the wild-type enzyme (manuscript in preparation). The presence of the threonine N-terminus was confirmed

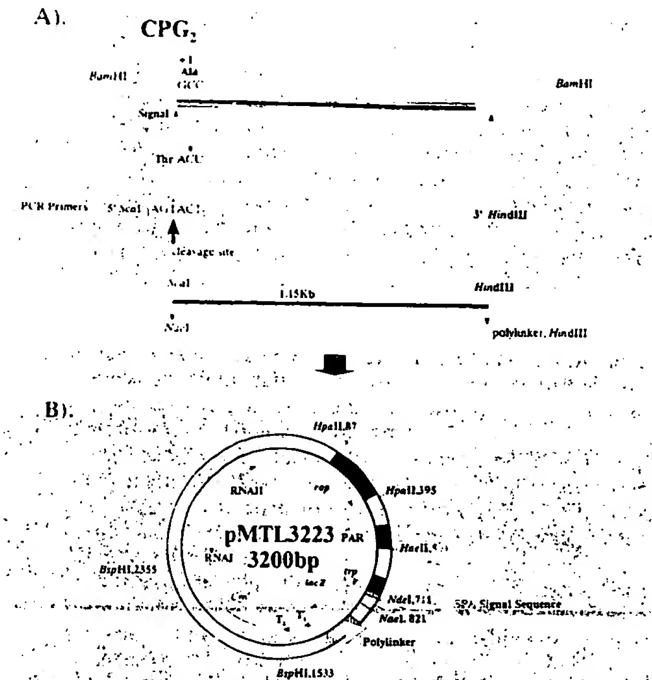

Figure 1. Insertion of the mutated CPG2 gene into an *E. coli* expression vector: A, the position of the mutagenic PCR primers to the CPG2 gene and the nucleotides involved in the mutation of Ala (wild-type) to Thr; B, restriction enzyme map of the expression vector pMTL3223 indicating the insertional sites of CPG2 gene.

by pulsed liquid phase N-terminal sequencing on an ABI 477 sequencer. The mass observed by ESMS was 41 727.29 Da ± 3.47 (expected 41 725.8 Da).

Periodate Oxidation of N-Terminal Threonine. The carboxypeptidase G2 mutant possessing N-terminal threonine was buffer-exchanged into freshly made 0.1 M NH_4HCO_3 by gel filtration on a fast desalting column or a NAP-5 column (Pharmacia). It was then concentrated on a Centriprep 10 concentrator (extensively washed; see General Methods) to about 8.4 mg/mL. All estimates of CPG2 concentrations and quantities given in this paper are based on optical density at 280 nm, assuming an absorption of 0.4 AU for a 1 mg/mL solution. A concentration of 8.4 mg/mL corresponds to about 200 μmol/L N-terminal Thr. Thirty equiv of methionine (0.2 M in water) were added followed by 10 equiv of periodic acid (20 mM in 0.1 M NH_4HCO_3). After 10 min at room tempe ature, the reaction was stopped by addition of 0.5 M 1,3-diaminopropan-2-ol (in 90% 1,4-butanediol) to give a final concentration of 50 mM. After a further 20 min at room temperature, the protein was separated from excess reagents and small molecular w ight aldehydes by gel filtration on a fast desalting column in 0.1 M sodium acetate pH 4.6 (0.1 M sodium acetate, pH adjusted to 4.6 with acetic acid). The aldehydic protein was then concentrated to 10 mg/mL and could be kept

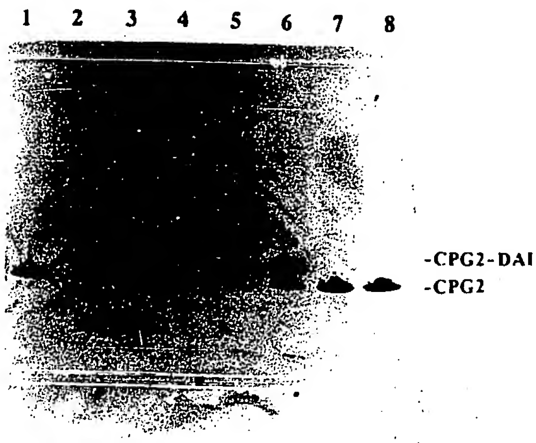

Figure 2. SDS–PAGE analysis of the oxidation of CPG2 and its conjugation with DAI-carbohydrazide: lane 1, 75 μM CPG2, 20 equiv of periodate, 10 min; lane 2, 78 μM CPG2, 10 equiv of periodate, 20 min; lane 3, 78 μM CPG2, 10 equiv of periodate, 10 min; lane 4, 78 μM CPG2, 10 equiv of periodate, 5 min; lane 6, 75 μM CPG2, 20 equiv of periodate, 10 min; lane 7, 80 μM CPG2 not oxidized incubated with 20 equiv of DAI–carbohydrazide; lane 8, 80 μM CPG2 not oxidized. Lanes 2–6: CPG2 was incubated with 20 equiv of DAI–carbohydrazide after oxidation.

at 4 °C from 1 to 14 days until needed for the conjugation. The mass of the aldehydic protein observed by ESMS was 41 713.44 ± 7.37 (see Discussion).

The reactivity of the aldehyde was checked by incubation of 1 to 2 μL of sodium acetate pH 4.6 containing about 0.1 nmol of oxidized CPG2 (8.4 μg of protein, 0.2 nmol of aldehyde as the protein is a homodimer (Sherwood et al., 1985)) with 4 μL of the same buffer containing 1 nmol per μL of des-Ala^{B30}-insulin carrying a carbohydrazide group on the C-terminus of the B chain (DAI–carbohydrazide). The DAI–carbohydrazide was prepared by reverse proteolysis as previously described (Rose et al., 1991). After 20 h at 37 °C, the samples were analyzed by SDS–PAGE, and the degree of conjugation between the oxidized enzyme and the insulin derivative gave a measure of the aldehydic reactivity of the former.

Digestion with Lysyl Endopeptidase. Four 1-mL portions of the A5B7 solution (see Materials) were buffer-exchanged into 50 mM Tris pH 8.4 (pH adjusted with HCl) by gel filtration on a fast desalting column and then concentrated on a Centriprep 10 to a final concentration of about 5 mg/mL (the concentration of A5B7 antibody and of its fragments was estimated from the optical density at 280 nm assuming an absorption of 1.25 AU for a 1 mg/mL solution). Lysyl endopeptidase (10 mg/mL in water) was added to give a 3% w/w enzyme–substrate ratio and the mixture incubated at 37 °C for about 20 h.

Ion-Exchange Chromatography. After centrifugation in a bench-top centrifuge to remove some precipitate the digest was diluted with 4 volumes of 50 mM sodium acetate pH 4.6 (adjusted with HCl) and loaded on a SP-trisacryl column (10- × 1.5-cm diameter) equilibrated in the same buffer. The column was washed with the same buffer and developed with a 400-mL salt gradient from 0 to 400 mM NaCl, still in the same buffer, at a flow rate of 0.34 mL/min. The fractions containing F(ab′)$_2$ were pooled, concentrated, buffer-exchanged into 10 mM Tris·HCl pH 8.0 on the fast desalting column, and concentrated again. The typical yield of this preparation was 35–40% of the theoretical maximum for F(ab′)$_2$.

Reverse Proteolysis. Solid carbohydrazide was added to the purified F(ab′)$_2$ (typically about 5 mg protein in 700 μL 10 mM tris pH 8.0) to get a final carbohydrazide concentration of 2.5 M. The pH was then lowered to 5.5 by addition of glacial acetic acid prior to addition of lysyl endopeptidase (10 mg/mL in water, enzyme:F(ab′)$_2$ ratio of 5% w/w). The amounts of carbohydrazide and acetic acid were calculated as follows: the sum of the volumes, in μL, of the F(ab′)$_2$ and of the enzyme solutions was multiplied by 0.265 to obtain the amount of carbohydrazide (in mg) and by 0.023 to obtain the amount of acetic acid (in μL) required. These quantities had been determined by tests on larger volumes without protein and take into account the volume increase due to carbohydrazide. The mixture was allowed to stand at room temperature for 3 h whereupon the reaction was stopped by addition of trasylol (100 mg/mL in water, 30-fold mass excess over the lysyl endopeptidase). The mixture was then gel-filtered on a Superose 12 column in 0.1 M sodium acetate pH 4.6 (adjusted with acetic acid) at a flow rate of 0.6 mL/min. The peak corresponding to F(ab′)$_2$ was collected in a tube containing Trasylol (10-fold mass excess over the quantity of peptidase used for the reverse proteolysis), concentrated, and made 10 mM in urea by adding 1 M urea in water. After 1 h at room temperature, the sample was desalted on a fast desalting column equilibrated in the same sodium acetate buffer. The protein recovery was quantitative.

The incorporation of carbohydrazide was quantified by incubating 100 μg of F(ab′)$_2$–carbohydrazide with 5 nmol of O=CHC$_6$H$_4$-*m*-CH=NOCH$_2$CO-ferrioxamine labeled with ^{55}Fe, as described by Fisch et al. (1992).

Conjugation (Hydrazone Formation). The F(ab′)$_2$–carbohydrazide obtained as described above was mixed with the aldehydic CPG2 (0.8 mg of CPG2 for 1 mg of F(ab′)$_2$–carbohydrazide, which corresponds to about four aldehyde groups per hydrazide). The mixture was concentrated in a Centriprep 10 concentrator to a final volume of about 700 μL and was then allowed to stand at room temperature for about 60 h, after which time it was gel filtered (in two portions) on Superose 12 in phosphate-buffered saline (PBS: 8 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L of Na$_2$HPO$_4$·2H$_2$O, 0.2 g/L of KH$_2$PO$_4$, pH 7.4) at a flow rate of 0.4 mL/min. The fractions containing the conjugate were pooled and concentrated in a Centriprep 10 concentrator. The conjugate can be stored at −20 °C. The yield of the conjugation was typically about 33%. The overall yield of the conjugate preparation was 10–15% based on starting IgG and 30% based on starting CPG2.

RESULTS AND DISCUSSION

Preparation of Aldehydic CPG2. An aldehyde group was introduced at the N-terminus of carboxypeptidase G2. The homodimeric mutant, which had threonine N-terminal, was subjected to mild oxidation at pH 8.3 (the pH of freshly prepared 0.1 M NH$_4$HCO$_3$) with 10 molar equiv of periodic acid per N-terminal Thr (i.e., 20 equiv per molecule of the dimeric enzyme). The presence of the aldehyde was detected by the formation of a hydrazone with DAI–carbohydrazide: after 20 h at pH 4.6 and 37 °C, analysis by SDS–PAGE shows that while in a nonoxidized control, the enzyme still migrates as a single band at 41 kDa (Figure 2, lane 7), reaction with oxidized enzyme leads to two bands (lane 2–6), the lower band representing less than 50% of the staining intensity, corresponds to the unconjugated enzyme, while the upper band corresponds to the CPG2–DAI conjugate. This result shows that N-termini of both subunits of CPG2 are accessible to the oxidation and hydrazone formation with DAI–carbohydrazide.

The enzymic activity of CPG2 was not altered by the oxidation of N-terminal threonine: the specific activity of the oxidized enzyme was found to be 126 U/mg which is not significantly different from the 131 U/mg of the unoxidized mutant enzyme. It is thus possible to introduce an aldehyde at the N-terminus of the CPG2 without altering its activity.

Both oxidized and unoxidized enzymes were analyzed by ESMS. For the unoxidized mutant enzyme, the observed mass (41 727.29 Da ± 3.47) was in agreement with the expected mass (41 725.8 Da). For the oxidized enzyme, the observed mass (41 713.44 Da ± 7.37) was significantly higher than the expected mass for the aldehydic protein (41 680.8 Da) and for the hydrated aldehyde (41 698.8), but was in agreement with the mass expected for the methanol hemiacetal of the aldehydic enzyme (41 713.8 Da). The hemiacetal was seen instead of the aldehyde because for ESMS analysis the samples were diluted to produce a solvent mixture containing water/methanol/acetic acid (49.5:49.5:1, by volume). The oxidation of the N-terminal Thr to an aldehyde could thus be achieved without affecting other groups in the protein.

Geoghegan and Stroh (1992) studied the oxidation of a peptide containing Tyr, His, Met, and Trp at slightly basic pH, such as we use: only methionine was seen to be affected, and that only to a very slight degree. Cysteine was not tested, but is not of interest here since CPG2 does not contain cysteine. To protect the six methionine residues contained in CPG2 (Minton et al., 1984), the oxidation was performed in presence of a 30-fold excess of methionine. There were thus five molecules of free methionine for each methionyl residue in the protein and 3 mol of methionine per mole of periodate added. This tactic has been shown to prevent oxidation of the Met residues of G-CSF under similar conditions to those described here (Gaertner et al., 1993).

It is important to avoid contamination of the oxidation solution by the glycerol present as a preserving agent on the concentrator membrane. The extensive washing procedure described is sufficient for this purpose; in that oxidation succeeded where it had previously failed without the wash.

In other experiments (data not shown), variations in protein concentration (40 to 200 μmol/L in terms of N-terminal Thr), and excess of periodate (5 to 20-fold) gave similar results in the conjugation test with DAI–carbohydrazide and had no effect on the enzymic activity of the oxidized enzyme. The procedure described in the Experimental section was found to work routinely leading to a conjugation with DAI–carbohydrazide close to 90% as determined by SDS–PAGE.

Preparation of F(ab′)$_2$–Carbohydrazide. The A5B7 antibody, which is a murine IgG1 κ, can be slowly digested to a F(ab′)$_2$ fragment with lysyl endopeptidase at pH 8.4. It is important to remove intact antibody at this stage because it is more difficult to remove it from the final conjugate. The F(ab′)$_2$ was thus purified by cation-exchange chromatography. After purification, the F(ab′)$_2$ was transferred to a buffer with low salt to avoid any interference of sodium ions (from the salt gradient) with the reverse proteolysis: sodium ions are known to inhibit cleavage by lysyl endopeptidase (K_i = 15 mM, technical information sheet from Wako) even if this effect has not been proven for reverse proteolysis.

Reverse proteolysis was then performed as described in the Experimental Section. The conditions are the optimal conditions of (Fisch et al., 1992). It is important to keep the F(ab′)$_2$–carbohydrazide, once isolated, in the presence of a large excess of Trasylol to avoid the action of traces of lysyl endopeptidase which would cleave the

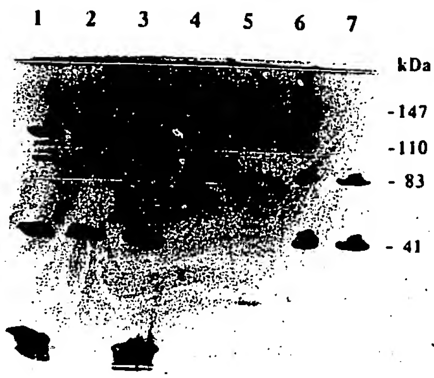

Figure 3. SDS–PAGE analysis of the conjugation of F(ab′)$_2$–carbohydrazide with oxidized CPG2: lane 1, F(ab′)$_2$–carbohydrazide with unoxidized CPG2; lane 2, control F(ab′)$_2$ (reverse proteolysis with Trasylol instead of lysyl endopeptidase) with oxidized CPG2; lane 3, F(ab′)$_2$–carbohydrazide with oxidized CPG2; lane 4, F(ab′)$_2$; lane 5, IgG; lane 6, purified conjugate; lane 7, oxidized CPG2 incubated with 1 equiv of carbohydrazide.

carbohydrazide once the conditions forcing the equilibrium in favor of synthesis (pH 5.5 and 2.5 M carbohydrazide) no longer remain.

Typically, the incorporation of carbohydrazide obtained with antibody A5B7 is about 0.5 carbohydrazide per F(ab′)$_2$, as determined by incorporation of ^{55}Fe labeled aldehyde. If, as expected, the distribution of F(ab′)$_2$, F(ab′)$_2$–carbohydrazide, F(ab′)$_2$–(carbohydrazide)$_2$ follows a binomial distribution, this corresponds to 56% of unmodified F(ab′)$_2$, 37% of F(ab′)$_2$–carbohydrazide, and 6% F(ab′)$_2$–(carbohydrazide)$_2$. The species we are most interested in is the monocarbohydrazide derivative because it should lead to a single conjugate with a monovalent partner (see later). The yield of this species would be optimal at 1 carbohydrazide per F(ab′)$_2$, but then the proportion of dicarbohydrazide derivative would be 25%. In principle, the unmodified F(ab)′$_2$ can be recycled (recoupled with carbohydrazide) but this was not attempted.

IgG, F(ab)′$_2$, and F(ab)′$_2$-carbohydrazide were analyzed by ESMS after reduction of disulfide bridges by dialysis in 10 mM dithiothreitol. In all three samples, two main signals were obtained corresponding to the light and heavy chain. The light chain had the same mass in all three samples (23 189–23 194 Da) indicating that the light chain is not modified by this procedure. The heavy chain mass was 26 416 Da in the F(ab)′$_2$ and 26 489 Da in the F(ab)′$_2$-carbohydrazide. The 73-Da difference between the two heavy chains is in agreement with the 72-Da increment expected by the incorporation of one carbohydrazide.

Conjugation. SDS–PAGE (see Figure 3, lane 3) shows that a conjugate with a mass of 147 kDa is produced when F(ab′)$_2$ after reverse proteolysis is incubated with 1 equiv of oxidized mutant CPG2 dimer (i.e., 4 aldehyde per hydrazide) at pH 4.6 for 60 h at room temperature. In contrast, no conjugate is formed when the same F(ab′)$_2$ is incubated with unoxidized CPG2 (lane 1) or if oxidized CPG2 is incubated with F(ab′)$_2$ that has been incubated in 2.5 M carbohydrazide as for reverse proteolysis but with the lysyl endopeptidase replaced by Trasylol (lane 2).

When analyzed by gel filtration, the conjugation mixture gives the profile shown in Figure 4a: a shoulder (A) and three peaks (B–D). Gel electrophoresis of these fractions (Figure 4b) shows that peak B corresponds to

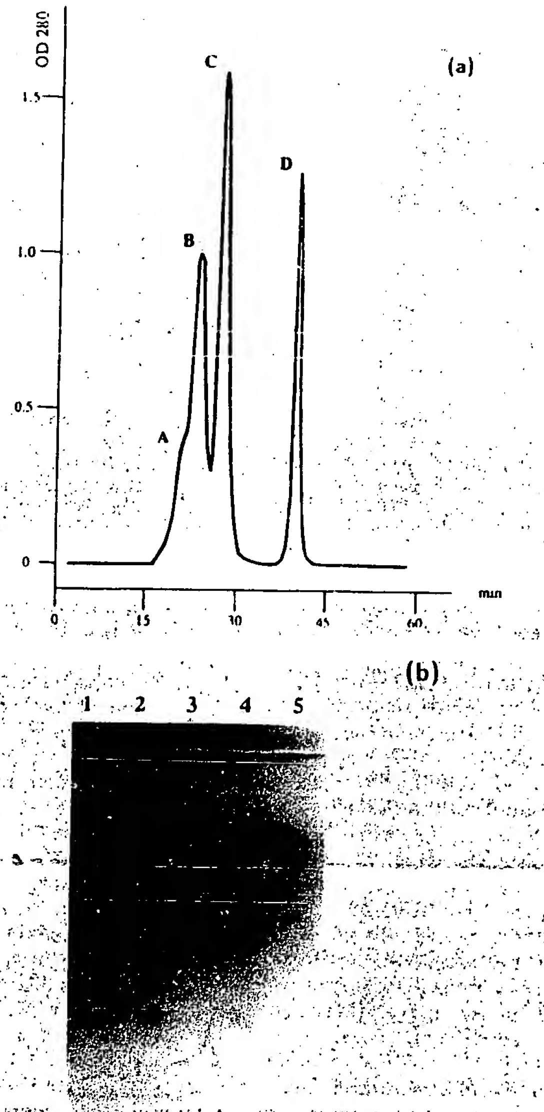

Figure 4. (a) Purification of the F(ab′)$_2$–CPG2 conjugate on Superose 12: A, shoulder containing aggregated material and conjugates of higher molecular weight; B, peak containing the F(ab′)$_2$–CPG2 conjugate; C, peak containing unreacted F(ab′)$_2$ and CPG2; D, Trasylol. (b) SDS–PAGE analysis of the Superose 12 fractions of the conjugation: lane 1, peak C; lane 2, peak B; lane 3, shoulder A; lane 4, CPG2; lane 5, F(ab′)$_2$.

the conjugate (lane 2: observed 147 ± 8 kDa, expected 153 kDa). Since one of the two enzyme subunits is noncovalently linked to the conjugate, the other major band in lane 2, corresponding to free enzyme, was to be expected. Peak C corresponds to unreacted F(ab)′$_2$ and CPG2 (lane 1). Electrophoresis for a shorter time (not shown) indicated that peak D contains the Trasylol (approx. 6 kDa) which was present to prevent the F(ab)′$_2$–carbohydrazide being converted back to F(ab)′$_2$ by residual traces of protease. The shoulder A (lane 3) also contained a little conjugate and we suppose that it might in addition contain more complex conjugates where, for instance, two F(ab)′$_2$ are linked to a CPG2 dimer (or two CPG2 dimers linked to one F(ab)′$_2$).

If one incubates the oxidized enzyme with 1 equiv of carbohydrazide at pH 4.6, a new band is seen, corresponding to an apparent M_r of 83 kDa (Figure 3, lane 7). The simplest explanation of this band is that two aldehydic subunits (42 kDa) have been covalently linked

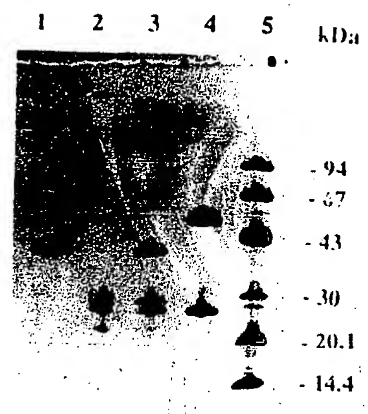

Figure 5. Characterization of the F(ab′)$_2$–CPG2 conjugate by SDS–PAGE: lane 1, CPG2; lane 2, reduced and carboxymethylated F(ab′)$_2$; lane 3, reduced and carboxymethylated conjugate; lane 4, reduced and carboxymethylated IgG, lane 5, reduced and carboxymethylated molecular weight markers.

through hydrazone bonds by a single carbohydrazide molecule. Since the new component elutes from Superose 12 under nondenaturing conditions at a position corresponding to approximately 160 kDa, the most likely hypothesis is that it consists of two dimers linked by a single covalent bond: dissociation in SDS would then give both the 83-kDA and the 42-kDa band, which is what is seen.

SDS–PAGE analyses of early preparations of the conjugate showed the same 83-kDa band to a quite significant extent (data not shown), and we concluded that some carbohydrazide had been introduced in the conjugate reaction mixture, having been noncovalently bound to the F(ab)′$_2$–carbohydrazide sufficient tightly to accompany it on gel filtration. The experiment described in the previous paragraph shows that very small quantities of carbohydrazide would be enough to promote the formation of the cross-linked byproduct. The noncovalent binding must be quite strong, since repeated gel filtration did not suffice to solve this problem. Therefore, based on the structural similarity between carbohydrazide (CO-(NHNH$_2$)$_2$) and urea, we sought to displace the carbohydrazide by pretreatment of the F(ab)′$_2$–carbohydrazide with a dilute solution of urea (10 mM). This operation had the hoped-for result, effectively abolishing the 83-kDa band in analyses of the conjugate. It was essential to carry out this urea treatment, since the cross-linked enzyme coelutes with the conjugate on gel filtration (data not shown) and it would have persisted as a contaminant.

Characterization of the Conjugate. The purified conjugate was also analyzed by reducing SDS–PAGE (Figure 5). On reducing SDS–PAGE (Figure 5 lane 3) the conjugate shows, as expected, four bands: one at 70 ± 5 kDa corresponding to the truncated heavy chain linked to a CPG2 subunit, a second at 41 kDa corresponding to the noncovalently bound CPG2 subunit, a third at 28 ± 2 kDa corresponding to the unconjugated truncated heavy chain (present in reduced F(ab′)$_2$, lane 2, but not in reduced IgG, lane 4), and a fourth at 26 ± 2 kDa corresponding to the intact antibody light chain (present in reduced F(ab′)$_2$ and in reduced IgG).

The majority of the conjugate appears to have the wanted structure of one F(ab′)$_2$ linked to one CPG2 dimer. On strong overloaded nonreducing gels, (a faint band seen at 185 kDa indicates some small quantities of larger structures (two molecules of F(ab′)$_2$ or two of CPG2 dimer). Given the fact that there are two aldehydic groups per CPG2 dimer (one per subunit) and some

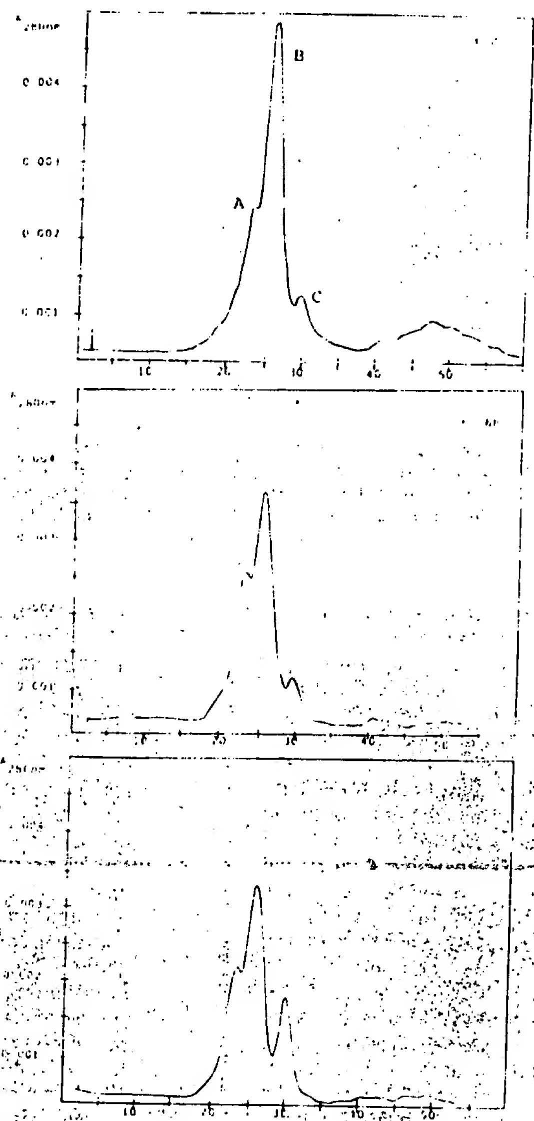

Figure 6. Stability of the $F(ab')_2$−CPG2 conjugate. The conjugate (1.4 mg/mL in PBS, pH 7.4) was incubated at 37 °C for the indicated time and then analyzed on Superose 12.

$F(ab')_2$ molecules have two carbohydrazide groups, it is not surprising to see them. That they are found in only relatively small amounts is probably due to steric hindrance between the close pairs of identical reactive groups.

Figure 6 shows the stability of the conjugate when incubated at 37 °C at pH 7.4. Peak B (the conjugate with the one $F(ab')_2$ linked to one CPG2 dimer stucture) diminishes slowly in favor of the shoulder, suggesting that an equilibrium exists between the wanted conjugate and conjugates of higher molecular weight. After 1 day or more, both peak B and the shoulder decrease in favor of peak C suggesting that the hydrazone bond is very slowly hydrolyzed. No significant changes are seen on SDS−PAGE after up to 24 h at pH 7.4 and 37 °C (data not shown). The rate of decomposition is slow enough not to be an obstacle to the use of the conjugate in ADEPT. Indeed, preliminary experiments in the nude-mouse xenograft system (data not shown) show a clear therapeutic effect of this conjugate.

As a confirmation of the proposed structure of the conjugate, Figure 7 shows that the bond between the

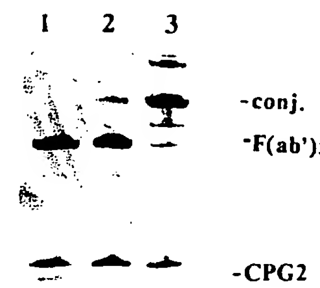

Figure 7. SDS−PAGE analysis of the digestion of the conjugate. $F(ab')_2$−CPG2 (1.2 mg/mL) was incubated with lysyl endopeptidase (1% w/w) in PBS (pH 7.4) at 37 °C: lane 1, 60 min; lane 2, 30 min; lane 3, undigested conjugate.

$F(ab')_2$ and the CPG2 is the one most susceptible to lysyl endopeptidase in the conjugate: it can be cleaved by 1% Achromobacter protease in 30 min before the CPG2 is degraded. Under these conditions, the $F(ab')_2$ is resistant to proteolysis.

The enzymic activity of the conjugate was found to be 45 U/mg conjugate which corresponds to 101 U/mg CPG2. This represents 75% of the activity of the unmodified mutant CPG2.

The binding activity of the conjugate was found to be 122 ± 43% (mean ± SD for 4 conjugate samples) in a competitive binding assay where the ability of the conjugate to compete with binding of an A5B7−alkaline phosphatase conjugate to CEA is compared with intact A5B7. Thus, the A5B7 $F(ab')_2$−CPG2 conjugate prepared by reverse proteolysis retains full binding activity. Similar conjugates prepared using conventional linker technology only retain approximately 70% binding activity in this assay suggesting that the head-to-tail conjugation described here leads to improved retention of antigen binding activity over conventional linker technology.

CONCLUSION

The linking of a large antibody fragment to a homodimeric enzyme can potentially yield a very complex mixture of products. Restriction of protein modification to the C-terminus of the heavy chain of the antibody fragment, and to the N-terminus of the enzyme subunit, limits the number of possible products and permitted us to prepare a head-to-tail immunoconjugate between a $F(ab')_2$-like fragment of the monoclonal anti-CEA murine IgG1 A5B7 and a mutant of carboxypeptidase G2 with retention of both the antigen binding and the enzymic activity.

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